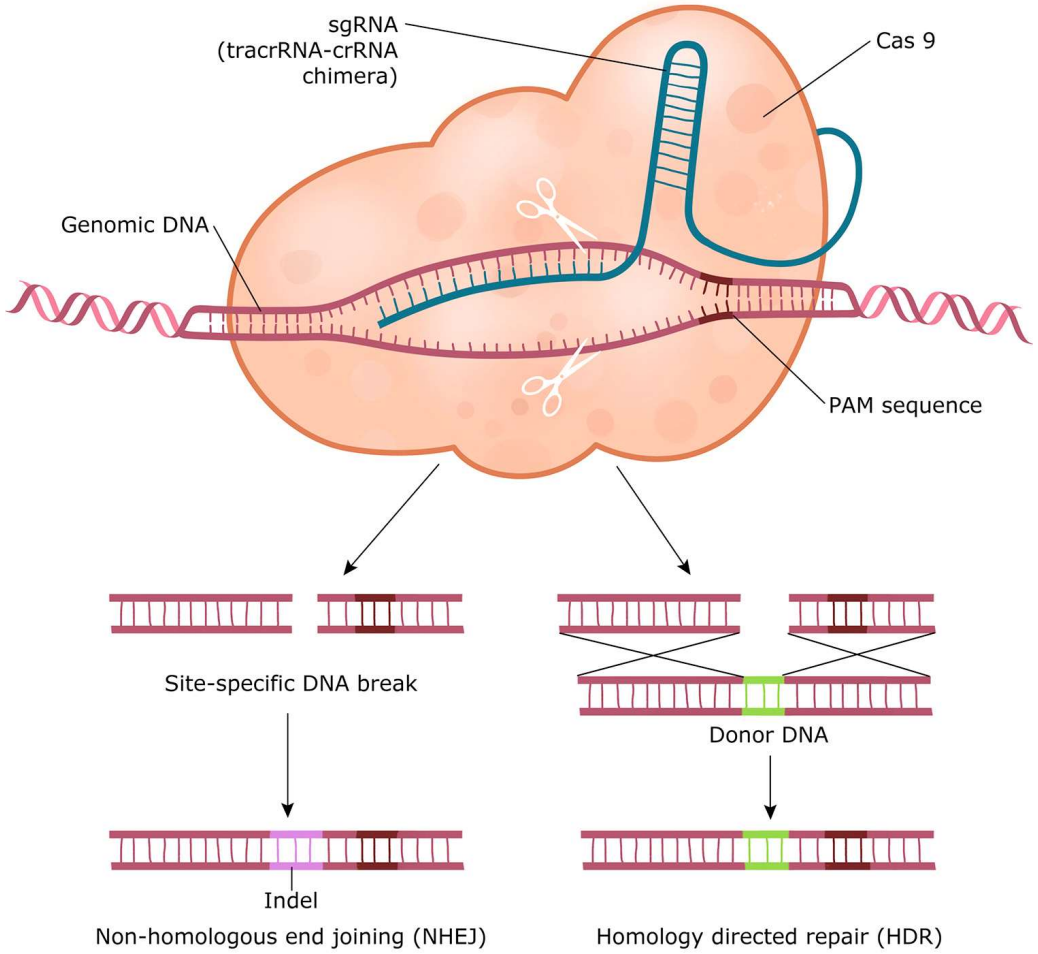
Literature Seminar #1

Intracellular delivery of CRISPR-Cas9 RNP for medical applications

211104

B4 Yuto Azumaya

CRISPR/Cas9 system



(Nataša Savić, Gerald Schwank,Translational Research,Volume 168,2016,15-21,)

- A tool that can perform DNA editing
- Consists of Cas9 protein and gRNA
- The gRNA recognizes and cleaves the target sequence.
- Cleaved sequences are repaired mainly by end joining (NHEJ) or homologous recombination (HDR)
- NHEJ is prone to errors such as deletions and mutations → suitable for gene deletions
- HDR can introduce the desired sequence depending on the design of the donor DNA.

Kim S, Broströmer E, Xing D, et al.*Science*. 2013;339(6121):816-819..
Ramirez-Phillips, A.C., Liu, D. *AAPS J* **23,** 80 ,2021.

Expectations for medical applications of the CRISPR/Cas9 system

Diseases for which CRISPR-Cas9 system application is expected

- Diseases caused by genetic abnormalities: Editing pathogenic genes for radical treatment

  Ex)
  - Huntington's disease: nucleotide elongation disorder, CAG repeat expansion of the huntingtin gene
  - Hemophilia: loss of clotting factors due to mutation
  - Duchenne muscular dystrophy: generation of loss-of-function proteins by frameshifting
  - sickle cell disease :deformation caused by a single Glu6Val mutation in the *HBB* gene

  etc.

- Viral diseases: Disrupting viral genes to inhibit their function

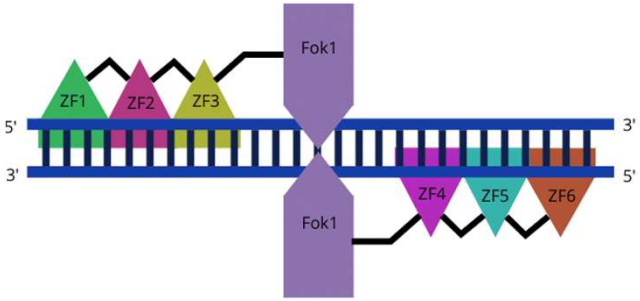
  Ex)
  - HSV
  - HIV
  - HPV
  - HBV

  etc.

- Cancer: Treat cancer by deleting oncogenes or introducing tumor suppressor genes

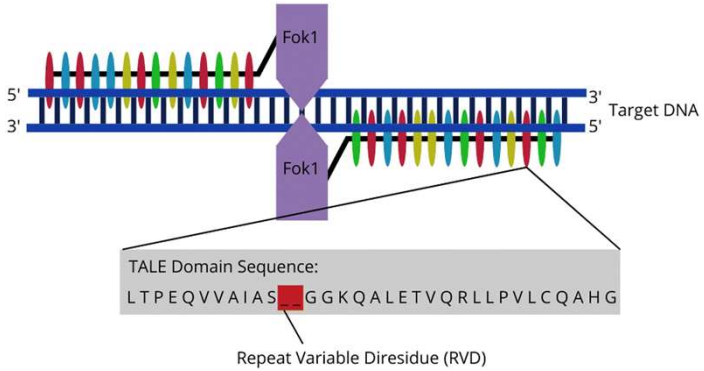
Ramirez-Phillips, A.C., Liu, D. *AAPS J* **23,** 80 ,2021.

other genome editing tools and advantages of CRISPR/Cas9



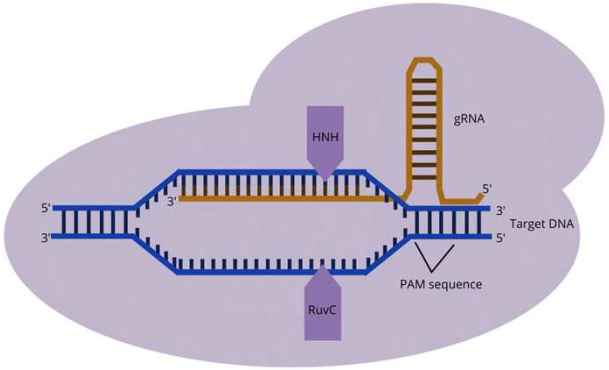
ZFN

Fok-1 nuclease
+ Zinc finger protein
(recognizes the target sequence)



TALEN

Fok-1 nuclease
+TALE domain (Base is recognized by amino acid sequence of RVD variable region)



CRISPR/Cas9

Cas9 nuclease
+ sgRNA(targeting genome)

・RNA : easily synthesizable & appliable to other genes by simply changing the RNA sequence.

sequence-specific DNA-binding protein
→difficult and expensive to engineer.

Ramirez-Phillips, A.C., Liu, D. *AAPS J* **23,** 80 ,2021.

Delivery form of CRISPR/Cas9 system

Large circular DNA

Long and short RNA

Large protein and short RNA

Cong-Fei Xu et al.Advanced Drug Delivery Reviews, Volume 168,2021,Pages 3-29,

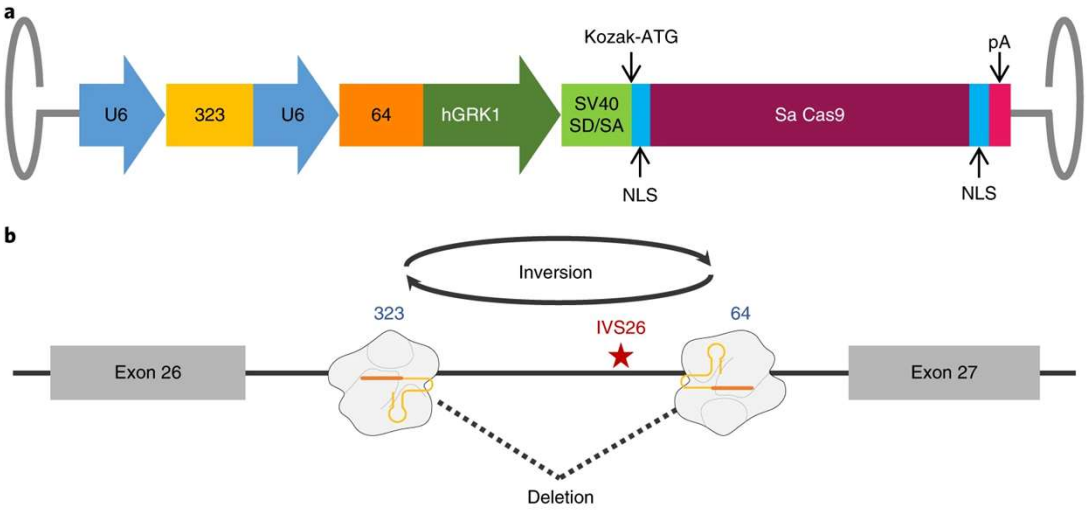
Investigational drugs using CRISPR-Cas9

EDIT-101

・Target disease: LCA10 (leber congenital amaurosis type 10)

・The IVS26 mutation in the CEP290 gene causes abnormal splicing, resulting in the addition of extra sequences to the mRNA → loss of function.

・Deletion or reversion of the mutation by cutting across the mutation site cures the splicing abnormality and allows normal CEP290 to be produced.



・Introduced by AAV in the form of pDNA

・Vitro: average productive editing rate (reverse + deletion) 16.6±6.5%

・Vivo: up to 28% in subretinal injection in cynomolgus monkeys (10% deletion thought to lead to visual recovery)

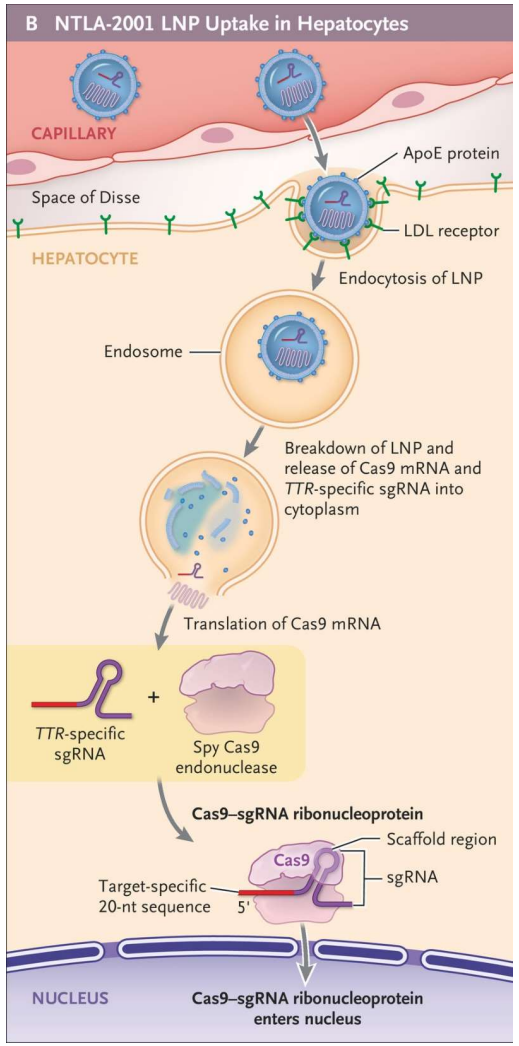
・Clinical trial (ongoing Phase 1, initial results): confirmed efficacy signal in 2 out of 3 patients in medium volume cohort

Maeder, M.L.,et al. *Nat Med* **25,** 229–233 (2019).

Investigational drugs using CRISPR-Cas9

- NTLA-2001
- Target disease: ATTR amyloidosis
- accumulation of misfolded transthyretin (TTR) protein →nerve damage or heart muscle disease
- TTR KO → decrease in the production of TTR

- lipid nanoparticle encapsulating Cas9 mRNA + sgRNA
- Introduced by intravenous infusion
- Vitro (primary human hepatocytes): Editing efficiency of more than 93.7%, decrease in the production of TTR of more than 95%, no off-target editing confirmed
- Vivo (cynomolgus monkeys): up to 73% editing efficiency, >94% serum TTR reduction, no off-target editing
- Clinical trial (on-going Phase 1, interim data): Sustained reduction in serum TTR protein concentration (up to 87%) was observed.



Gillmore, J. D., Gane, et al. *New England Journal of Medicine*, *385*(6), 493–502.

Delivery form of CRISPR/Cas9 complex

Large circular DNA

・stable
・simple to produce
・easy to deliver
(ex vivo with physical methods)
(in vivo with viral vectors)

・Long-term Cas9 expression by transcription and translation
→off-target DNA cleavage / host immune responses
・Possibility of insertional mutation

Long and short RNA

・limitation of the duration of Cas9 activity
→Reduction of off-targets
・Does not cause insertional mutation

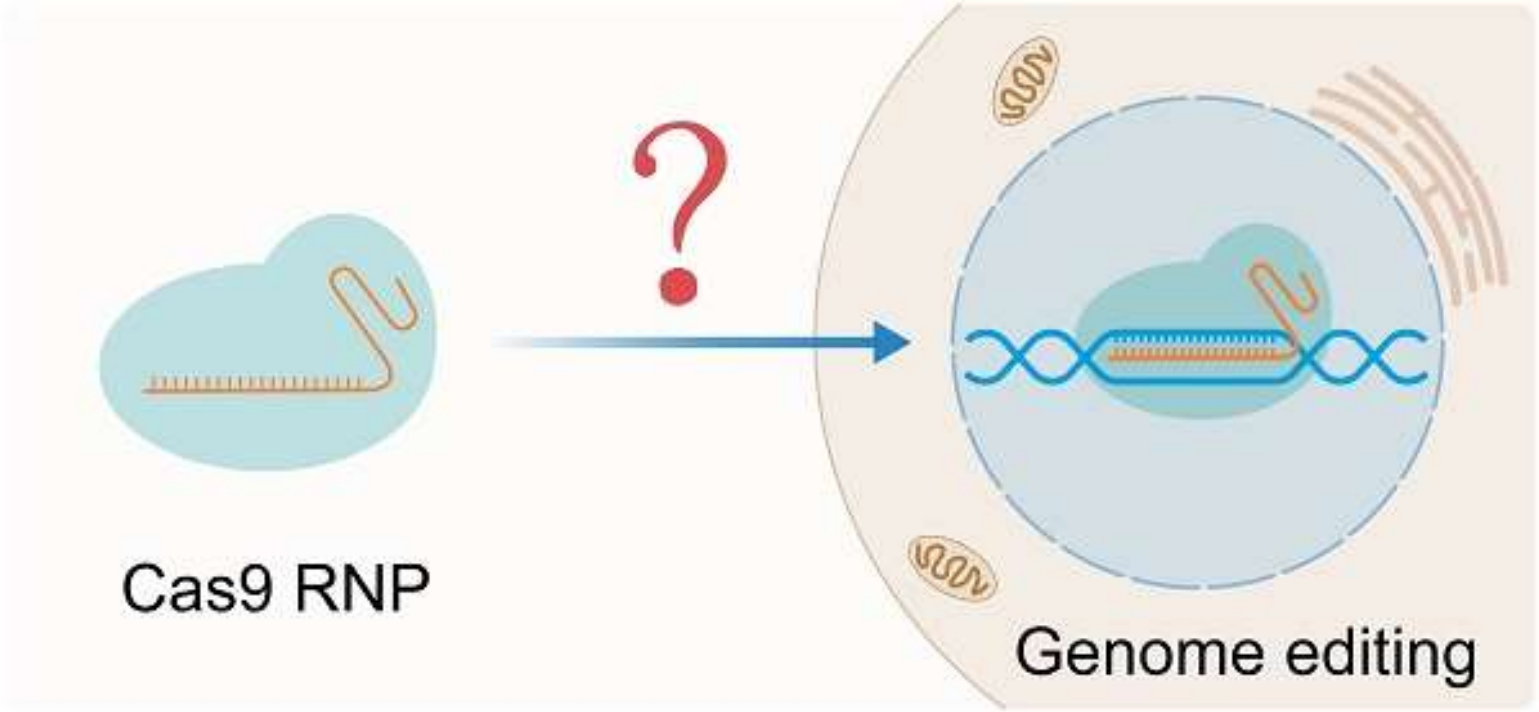
・Low stability of mRNA
・Requiring Translation

Large protein and short RNA

・No transcription and translation
→Editing is possible even for cells with low transcriptional and translational activity.
・Easy to control the amount of transfection
・Quick editing
・High activity can be expected. (binding to sgRNA is not inhibited)
・Lowest possibility of off-target cleavage
・Reduction of immune response
・No insertional mutation

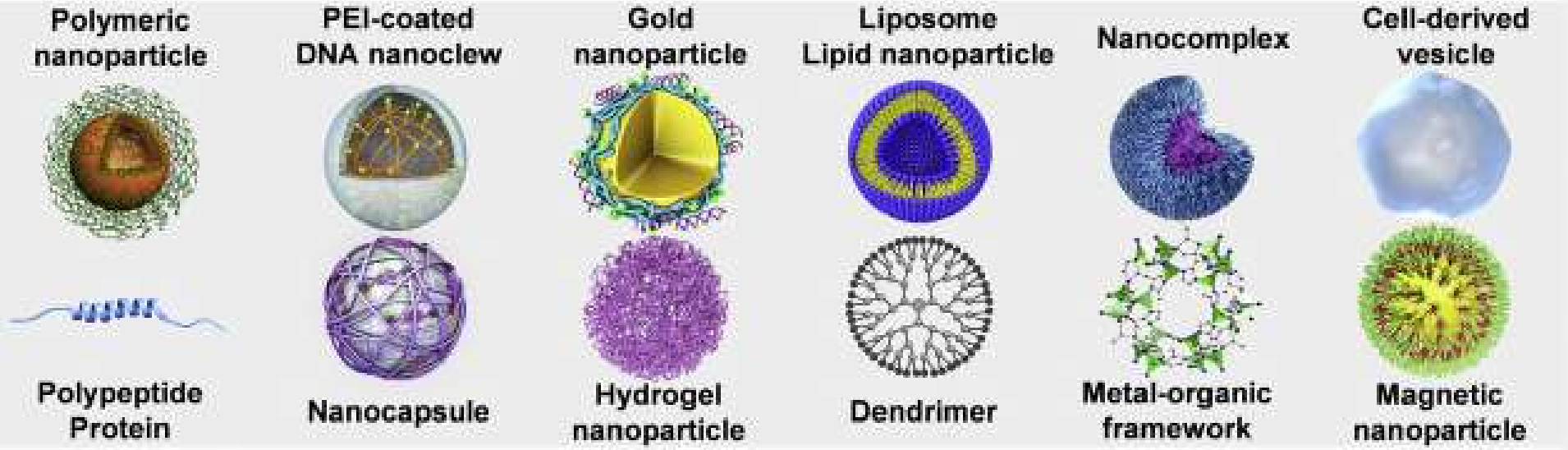
Cong-Fei Xu et al.Advanced Drug Delivery Reviews, Volume 168,2021,Pages 3-29,

How to deliver?

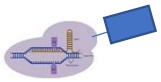


Zhang S, Shen J, Li D, Cheng Y. *Theranostics* 2021; 11(2):614-648.

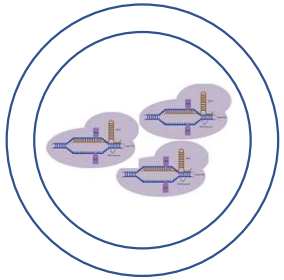
Various methods of delivering CRISPR/Cas9 RNP



Basic design concepts



or



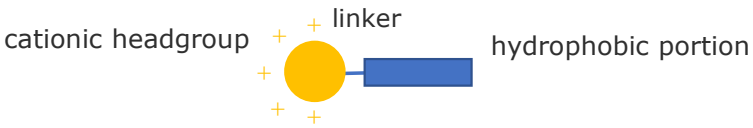
↓ Form nanoparticles
↓ Coating or attaching with membrane permeable molecules

- Membrane permeability: cationic lipids, PEI, CPP, etc.
- Nanoparticle formation: polymer, metal, DNA, etc.

Cong-Fei Xu et al.Advanced Drug Delivery Reviews, Volume 168,2021,Pages 3-29,

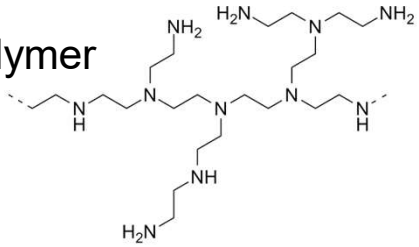
Cationic substances commonly used for membrane permeation

・ Cationic lipids



・PEI

Polyethyleneimine, a polymer



・ CPP

Peptides with cell membrane permeability
Many of them contain a large number of cationic residues.

Table 1: Overview of some the most commonly used CPPs describing their sequence, class and charge

Name	Amino acid sequence	CPP class	Charge
TAT	YGRKKRRQRRR	cationic	8
penetratin	RQIKIWFQNRRMK WKK	cationic	7
R9	RRRRRRRRR	cationic	9
MPG	GALFLGWLGAAGSTMGAPKKKRKV	amphipathic	24
Pep-1	KETWWETWWTEWSQPKKRKV	amphipathic	2
transportan-10	AGYLLGKINLKALAALAKKIL-amide	amphipathic	4
PepFect6	stearyl-AGYLLGK(ε-TMQ)INLKALAALAKKIL	amphipathic	10
Bac7	RRIRPRPPRLPRPRPRPLPFPRPG	proline-rich	9

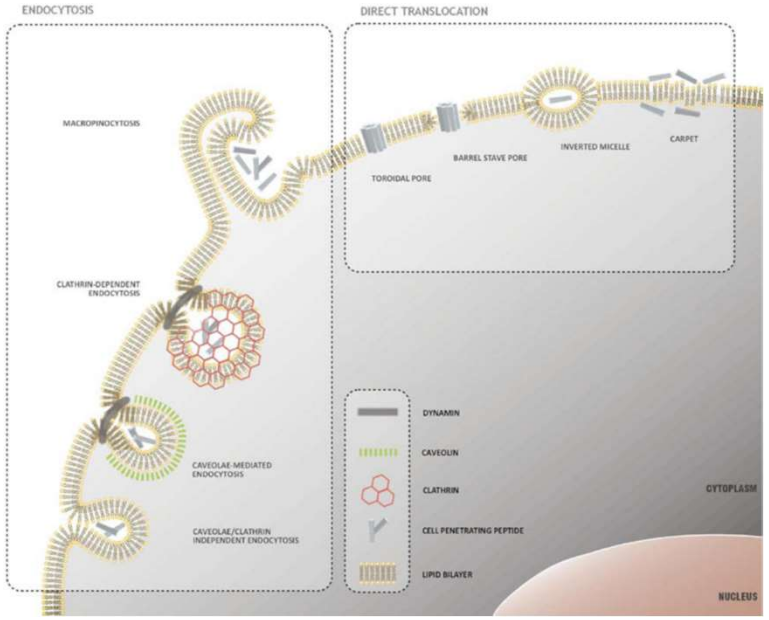
In both cases, uptake occurs by the following mechanisms

・Interaction with membranes

→Endocytosis

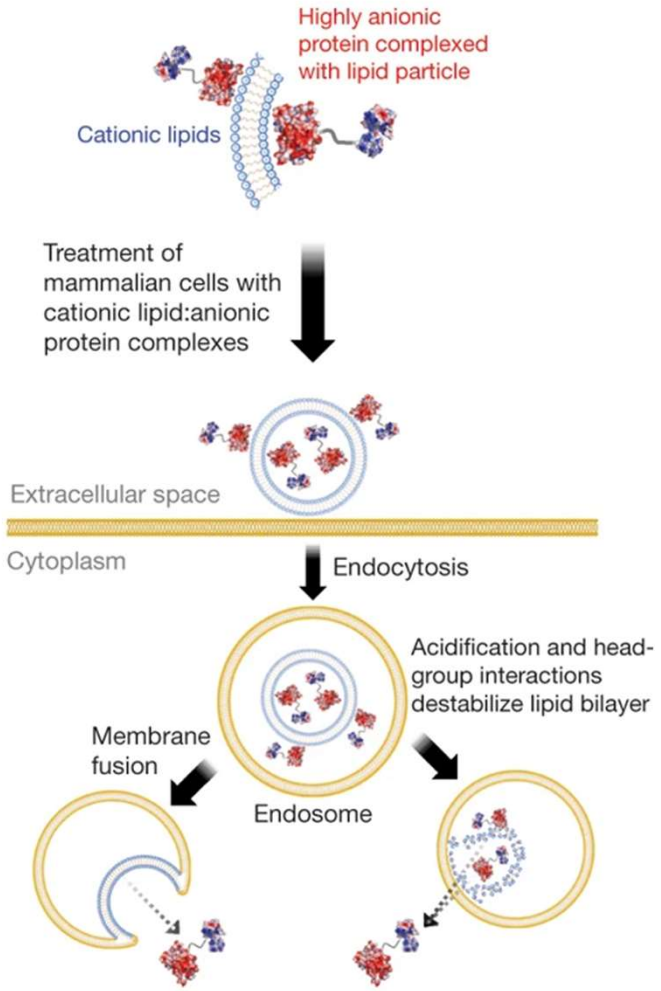
→Escape from endosomes

※All of them are cytotoxic.



Ruseska, I.; Zimmer, A. *Beilstein J. Nanotechnol.* **2020,** *11,* 101–123.

Methods of delivering CRISPR/Cas9 RNP ①: Synthetic lipid nanoparticles



・the Cas9/sgRNA RNP is negatively charged
→Encapsulated by lipid molecules in liposomes.

・Most commonly used in research applications.

Ex) lipofectamine CRISPRMAX
(Commercially available reagents dedicated to CRISPR transfection, the genome editing efficiencies achieved 55%, 75% and 85% in human iPSCs, mouse ES cells and HEK293FT cells)

・HDR achieved

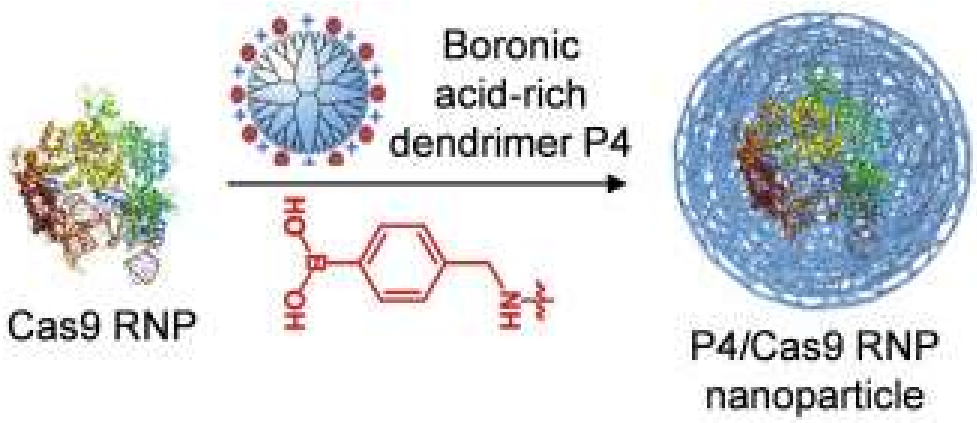
Zuris, J., Thompson, D., Shu, Y. *et al. Nat Biotechnol* **33,** 73–80 (2015).

Methods of delivering CRISPR/Cas9 RNP ② : polymers

- Form complexes with Cas9 RNPs using polymers

  Advantages
  - Easy to synthesize
  - Flexible structure and composition
  - Easy to functionalize
  - Degradable

Ex) PBA-rich dendrimer



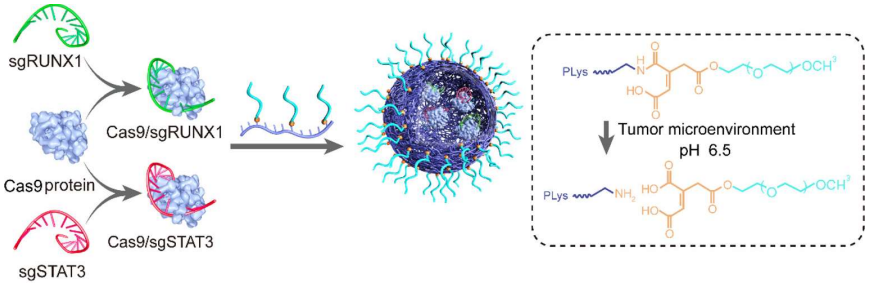
Interaction of phenylboronic acid (PBA) with proteins to form a more stable complex

- 20~40% in del (293T-EGFP cells)

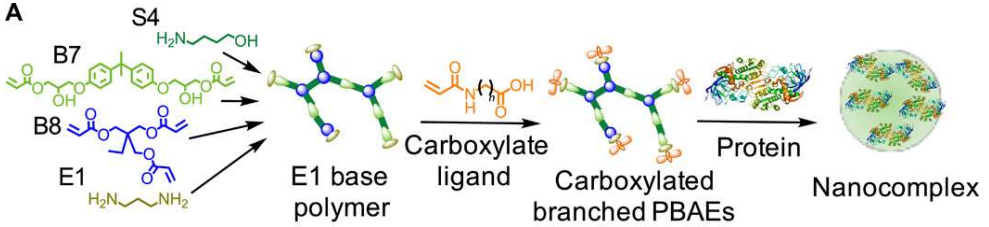
Zhang S, Shen J, Li D, Cheng Y. *Theranostics* 2021; 11(2):614-648.

Methods of delivering CRISPR/Cas9 RNP ② : polymers

PEGylated PLL

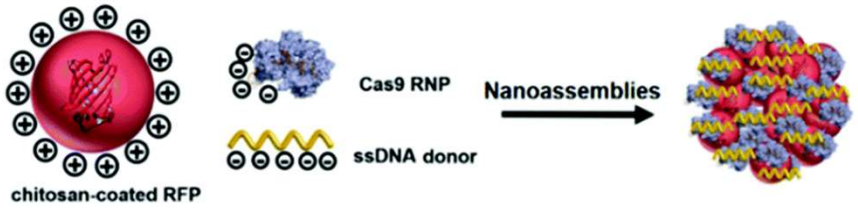


PBAE(poly(β-amino ester) nanoparticles)



・HDR achieved

Chitosan (CS) nanoparticles

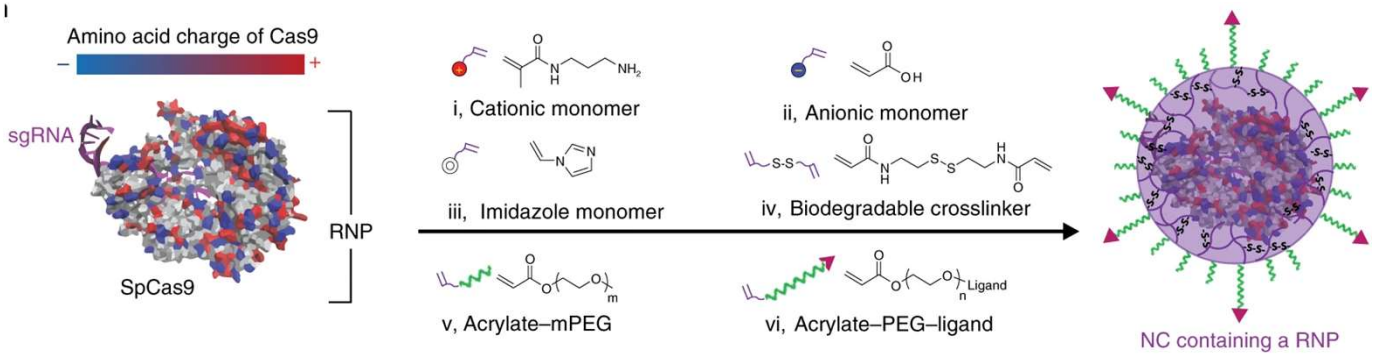


・HDR achieved

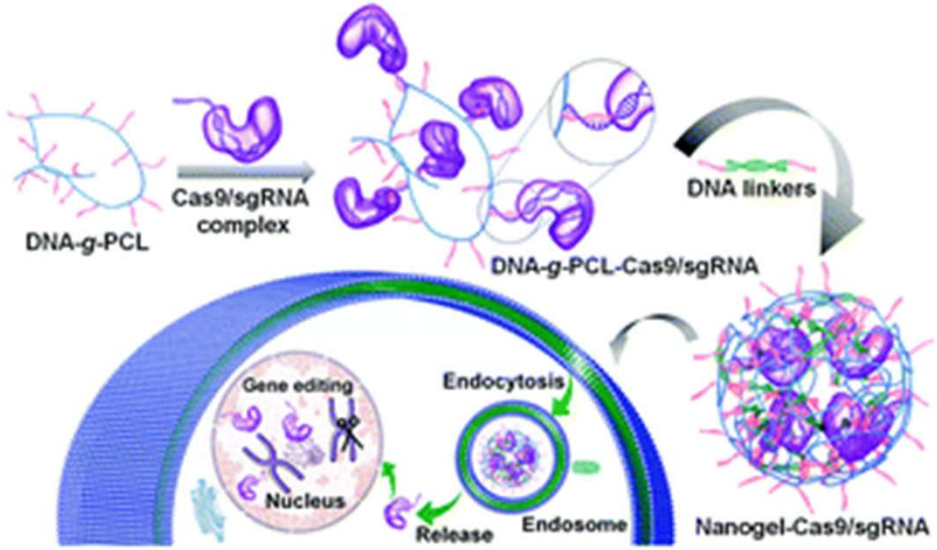
Zhang S, Shen J, Li D, Cheng Y. *Theranostics* 2021; 11(2):614-648.

Methods of delivering CRISPR/Cas9 RNP ③ :nanogel

nano capsule



G. Chen, A.A. Abdeen, et al. Nat. Nanotechnol., 14 (2019), pp. 974-980



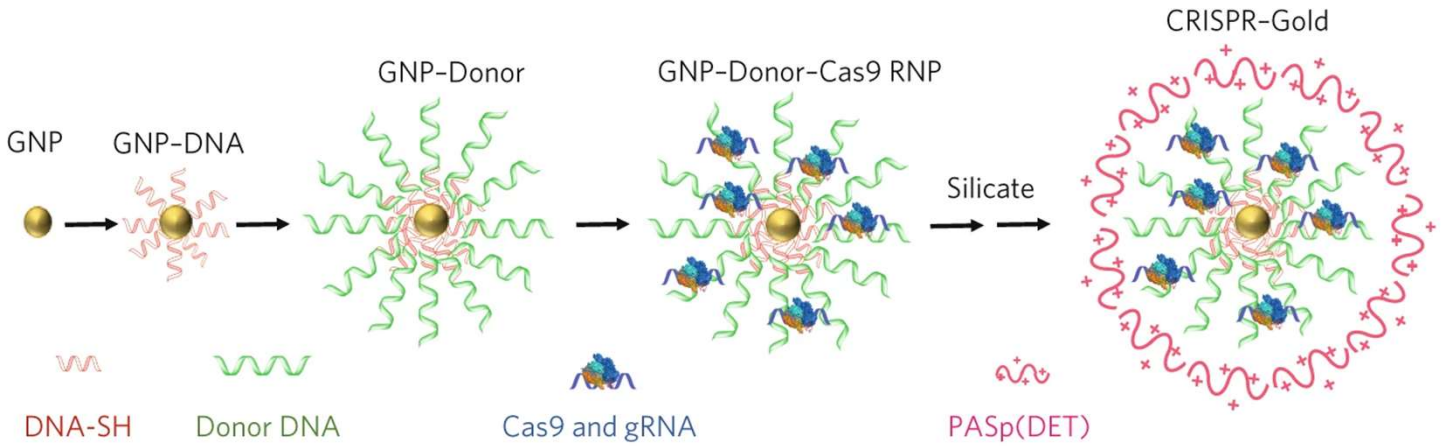
・18.7% in del

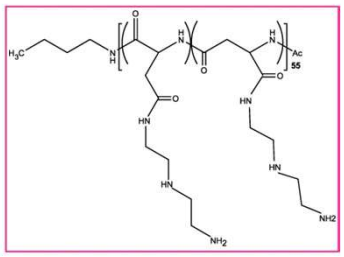
Zhang S, Shen J, Li D, Cheng Y. *Theranostics* 2021; 11(2):614-648.

Methods of delivering CRISPR/Cas9 RNP ④ : Inorganic nanoparticles

- Use inorganic particles, such as metals, to form the complex.

AuNP



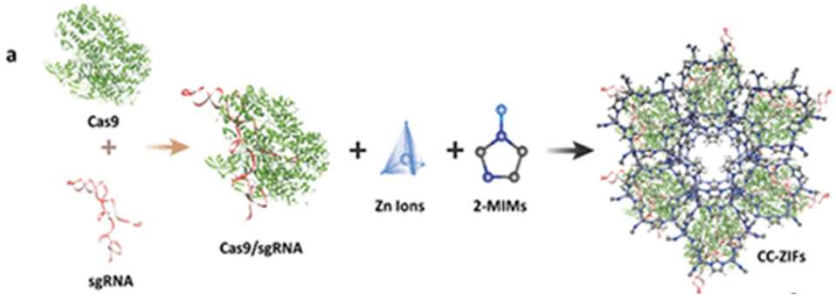


・HDR achieved(~5%)

Lee, K., Conboy, M., Park, H.M. *et al.Nat Biomed Eng* **1,** 889–901 (2017)

Methods of delivering CRISPR/Cas9 RNP ④ : Inorganic nanoparticles

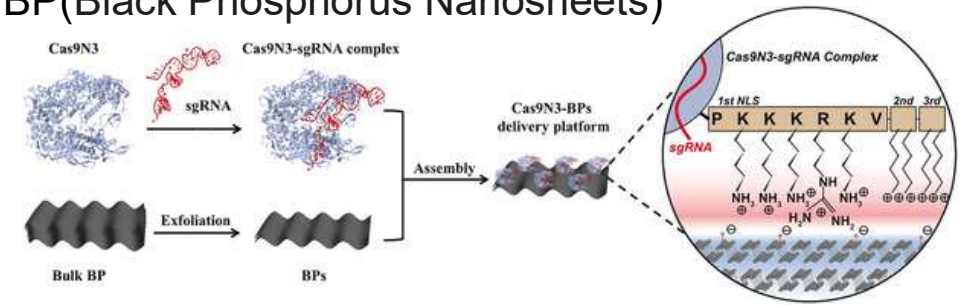
MOF(Metal-organic frameworks)



・30~60% in del
・HDR achieved

Shahad K. et al.
Journal of the American Chemical Society **2018** *140* (1), 143-146

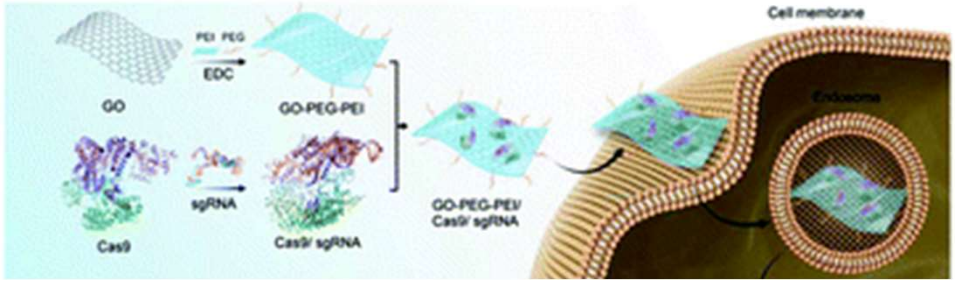
BP(Black Phosphorus Nanosheets)



・17~32% in del

W. Zhou, H. Cui, L. Ying, X.-F. Yu, *Angew. Chem. Int. Ed.* **2018**, *57*, 10268.

GO(graphene oxide)



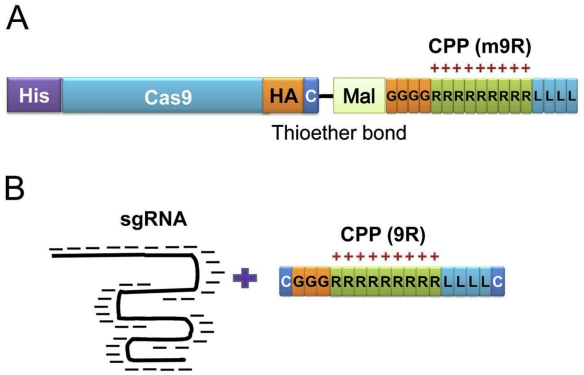
・30~60% in del

Yue, H., Zhou, X., Cheng, M., & Xing, D. (2018). *Nanoscale*, *10*(3), 1063–1071.

Zhang S, Shen J, Li D, Cheng Y. *Theranostics* 2021; 11(2):614-648.

Methods of delivering CRISPR/Cas9 RNP ⑤ : CPP conjugation

ex：Cas9 +CPP / CPP・sgRNA complex

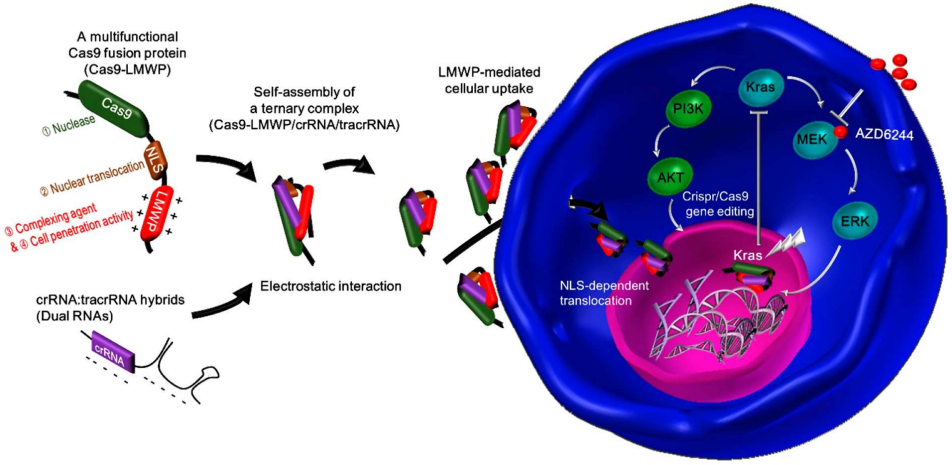


Cas9-SmaI-CPP
+sgRNA-CPP complex

16% in del (HEK293T *in vitro)*

Ramakrishna, S., Kwaku Dad, A. B., et al. *Genome Research*, *24*(6), 1020–1027.

ex： Cas9-LMWP-sgRNA ternary Cas9 RNP



Cas9-NLS-LMWP(CPP) protein
+sgRNA

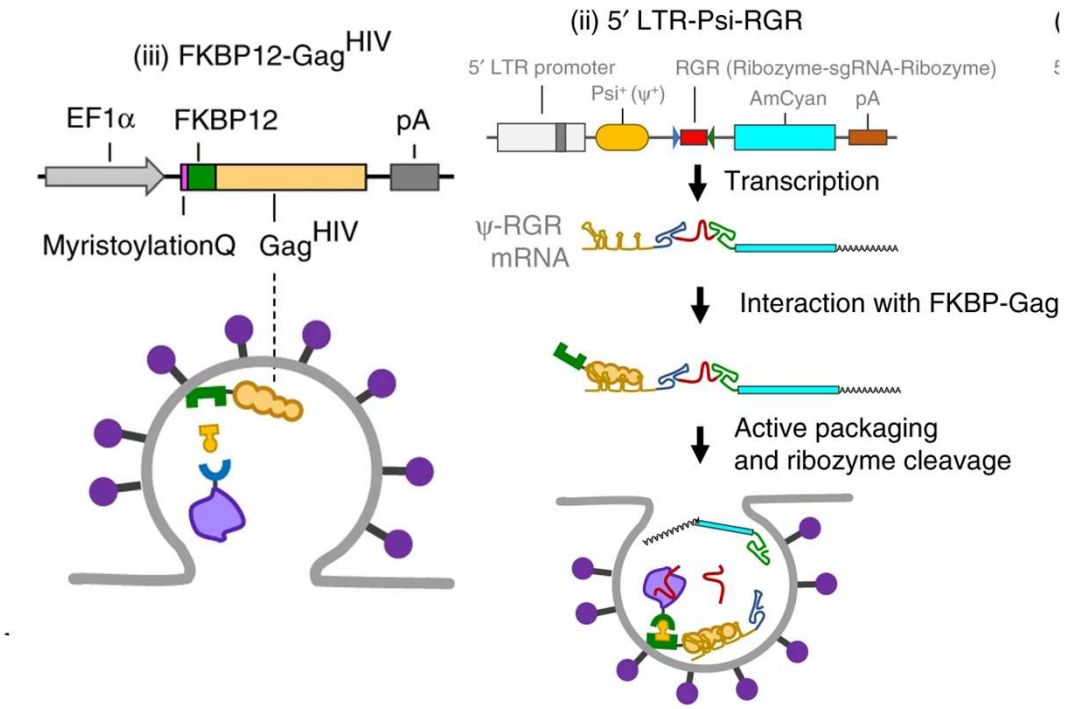
43.9% in del (A549 cells *in vitro)*

Seung Min Kim, et al. *ACS Nano* **2018** *12* (8), 7750-7760

Methods of delivering CRISPR/Cas9 RNP ⑥ : Biological Production

- Viral vectors, extracellular vesicles, etc.

Ex)NanoMEDIC



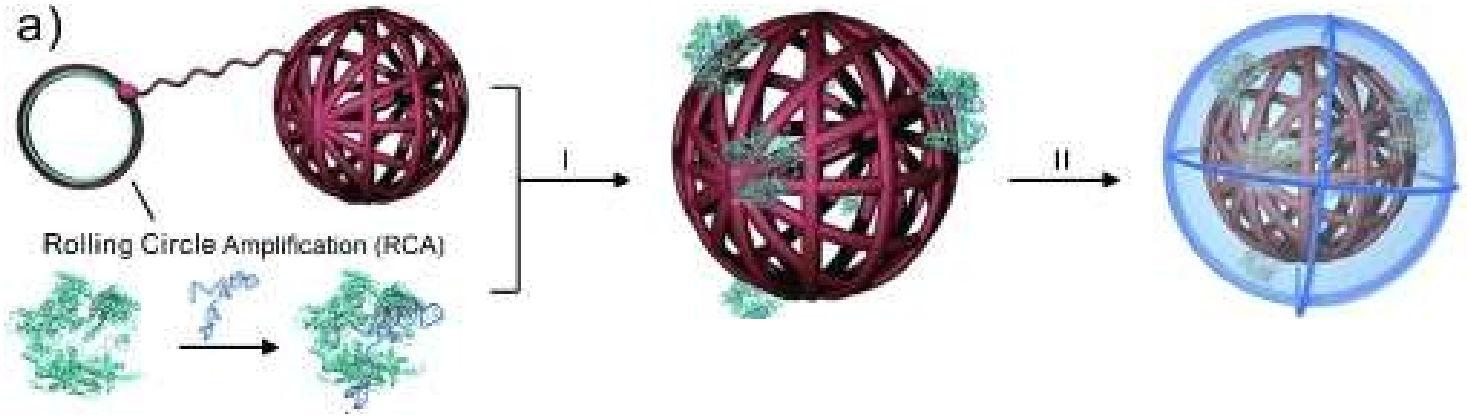
・Expression of three viral proteins (VSV, HIV-Gag, and LM) to form virus-like vesicles
・Cas9 protein is incorporated into EVs through interaction between FRB and FKBP
・sgRNA is incorporated into EVs by interaction between GAG and ψ⁺.
・Introduced into target cells by viral proteins on budding extracellular vesicles

・up to 92% exon skipping(DMD patient iPSCs)

Gee, P., Lung, M.S.Y., Okuzaki, Y. *et al. Nat Commun* **11,** 1334 (2020).

Methods of delivering CRISPR/Cas9 RNP ⑦ : DNA-

・DNA nanoclew



Short summary

- Various delivery methods for CRISPR-Cas9 RNPs have been developed.
- Many of them form nanoparticles or covalent complexes that are permeable to cell membranes and are transferred into cells via endocytosis.
- Nanoparticle materials are diverse(Lipids, polymers, inorganic particles, peptides, etc.)

Difficulty in causing HDR

Most of the existing systems have focused on gene deletion by NHEJ, and few have achieved gene transfer by HDR.

・HDR achieved

- ・liposome：5~85% in del/ 8~16% HDR
- ・AuNP ：14.6~34% in del / ~5% HDR
- ・ MOF ：30~60% in del / 20~% HDR
- ・Chitosan (CS) nanoparticles ：16.9~55.8% in del /12.5 % HDR
- ・PBAE(poly(β-amino ester) nanoparticles) ： 47~77 % in del / 4% HDR

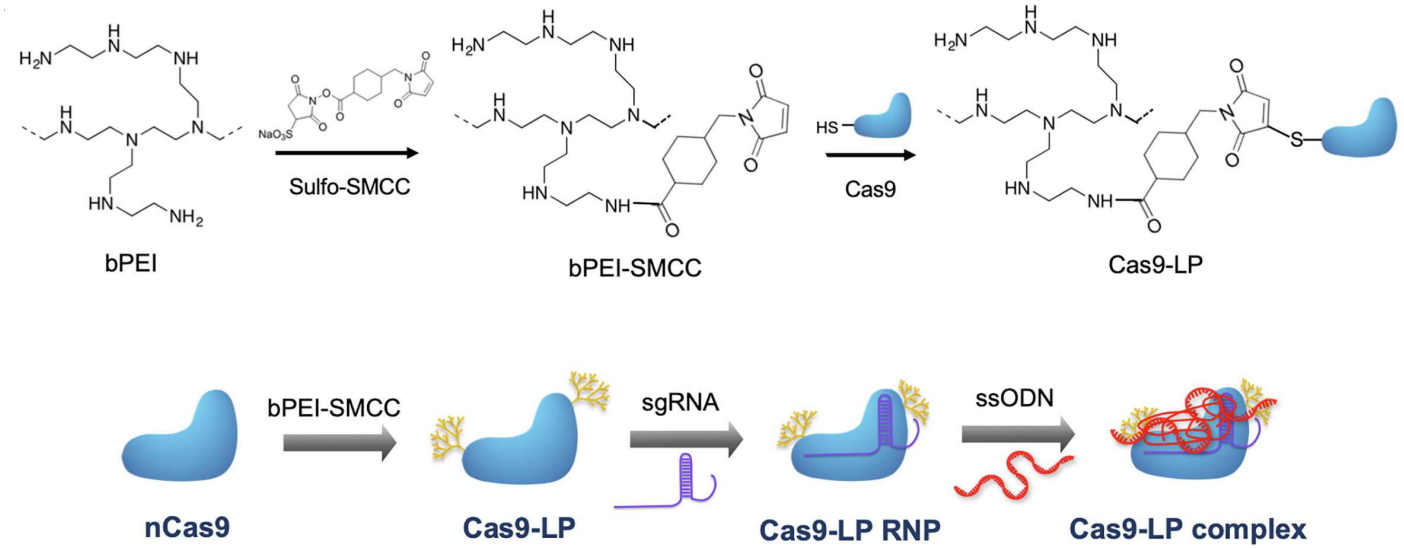
HDR is difficult to achieve with existing systems

・Low efficiency compared to NHEJ → Cytotoxicity of carriers (especially cationic career) occurs when trying to increase the concentration

・Co-localization of donor DNA is required(NHEJ：only RNP is required)

Zhang S, Shen J, Li D, Cheng Y. *Theranostics* 2021; 11(2):614-648.
Yoo Kyung Kang, et al.
Journal of Industrial and Engineering Chemistry,Volume 102,2021,Pages 241-250,

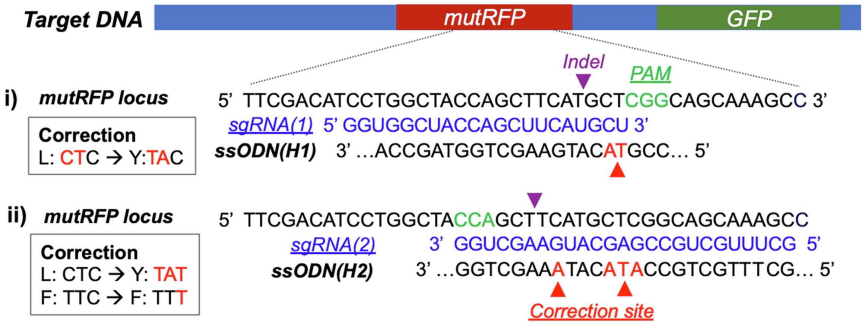
Challenge to create a system that can generate HDR with high yield

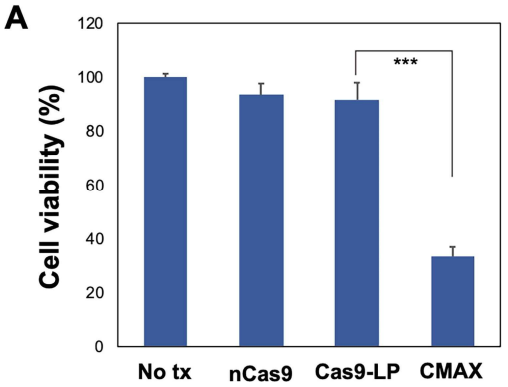


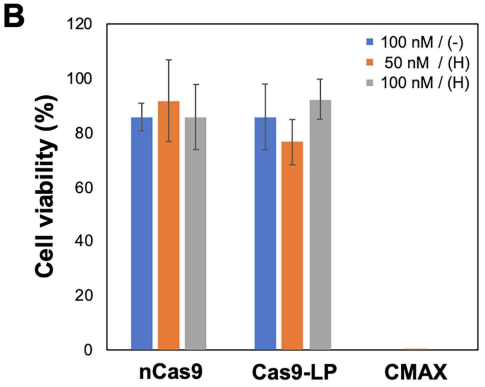
- As few carriers as possible → Aim to reduce toxicity
- Interaction and complexation with ssODN by charge
  →Enables co-localization with donor DNA

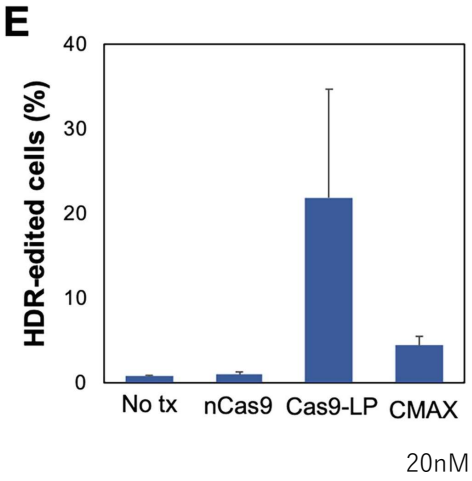
Yoo Kyung Kang, et al.
Journal of Industrial and Engineering Chemistry,Volume 102,2021,Pages 241-250,

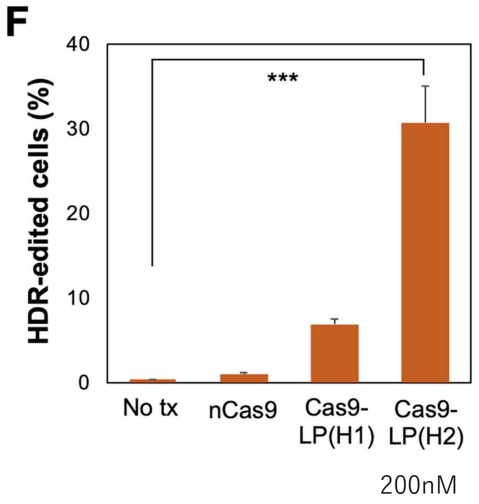
Challenge to create a system that can generate HDR with high yield











- Low cytotoxicity

→Higher concentration could be achieved

- HDR : up to 31% (exceeding CRISPRMAX)

- Still limited to use in vitro → awaiting vivo application

Yoo Kyung Kang, et al.
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Summary

introduction

- CRISPR-Cas9 is attracting attention as a powerful tool for genome editing therapy.
- Drug discovery using Cas9 has already begun, and some have advanced to clinical trials.
- Delivery of Cas9 in the form of RNPs has many advantages over other methods, and delivery methods are being developed to achieve these advantages.

Methods of delivering CRISPR/Cas9 RNP

- Intracellular delivery of CRISPR-Cas9 RNPs is generally performed by linking them with membrane-permeable materials.
- The materials of the complexes can be lipids, polymers, inorganic particles, peptides, etc.
- Most of the currently developed delivery methods target only deletion editing, and only a limited number of systems are capable of HDR-mediated gene transfer, but the development of delivery methods that enable this is underway.